

From the DEPARTMENT OF MEDICINE, SOLNA
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**NEW APPROACHES FOR DIAGNOSTICS AND THERAPY
OF ALLERGY TO PETS**

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New approaches for diagnostics and therapy of allergy to pets

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To my family, future, present & past

Just one more year and then you'd be happy

Gerry Rafferty "Baker Street"

In tribute to my time in London living next to Baker street and to the music in our L2:04 lab

ABSTRACT

Allergic diseases have been described already in 900 AD but today allergic diseases have reached epidemic proportions. About 30% of the world population is affected. Typical allergic symptoms of the immune system's reaction to allergens are runny nose, red eyes or skin reactions like itching, eczema, urticaria, as well as more severe symptoms like asthma and anaphylactic reactions. The overall aim of this thesis was to identify and characterize new pet allergens and to use these to improve diagnostics and prediction of pet allergy. Furthermore, to provide a relevant platform, a mouse model reflecting chronic asthma, for the development of novel treatment strategies for cat allergy.

In the first paper we studied dog saliva as a source of new allergen molecules for improved diagnostics of allergy to dog. We show that there are at least 12 protein bands in dog saliva that are recognized by IgE antibodies from dog-allergic patients. Several of those bands were not identified in dog dander extract. Furthermore, we demonstrate that about one-fifth of patients with symptoms to dog, but lacking IgE antibodies to dog dander, were IgE positive to saliva. Dog saliva was shown to be a significant allergen source that should be taken into account for improved diagnostics of dog allergy. Combining dog dander and dog saliva or spiking dog dander extract with dog saliva would be beneficial for developing enhanced dog allergy diagnostics.

In the second paper of this thesis we investigated the prevalence of sensitization to the novel cat allergen Fel d 7 in 94 cat-sensitized patients and elucidated Fel d 7's allergenicity and cross-reactivity with the homolog major dog lipocalin allergen Can f 1 on an epitope level. More than a third of the Swedish cat dander-sensitized patients, 39%, were IgE positive to Fel d 7 and we could show that Fel d 7 is a biologically active allergen. Our results demonstrate that Fel d is cross-reactive with Can f 1 and indicate that Fel d 7 has epitopes in common with Can f 1 which contributes to the co-sensitization observed in patients with allergy to cat and dog. Also, Can f 1 peptides spanning the Can f 1 sequence were used to map Fel d 7 binding epitopes in a 3D model based on the known structure of a human lipocalin homolog.

Paper III describes the association between sensitization patterns to individual cat and dog allergen molecules during childhood and symptoms to these furry animals up to 16 years of age. We investigated sensitization to individual cat and dog allergen molecules in childhood through adolescence using the BAMSE (Barn/Children Allergy/Asthma Milieu Stockholm Epidemiologic study) birth cohort. Sera and questionnaire data from 779 randomly collected children at 4, 8 and 16 years were examined. IgE reactivity to cat and dog allergen molecules were analyzed with the MeDALL (Mechanisms for the Development of ALLergy) chip. This is the first study to elucidate the usefulness of analyzing the individual cat and dog allergen molecules as predictors of cat and dog allergy development from childhood to adolescence. We report that IgE to Fel d 1 is as good as IgE to cat extract for diagnosis of cat allergy and IgE to Can f 1 is superior to IgE to dog allergen extract for diagnosis of dog allergy. Thus, molecular-based allergy diagnostics may offer new opportunities for improving diagnosis of pet allergy and in particular allergy to dog.

The last paper presents a relevant model for cat allergen-induced asthma in mice, exhibiting features of human chronic disease. Female BALB/c mice were presensitized with rFel d 1 adsorbed to Alum and subsequently challenged intranasally (i.n.) with cat dander extract (CDE) three consecutive days per week during five weeks. The new animal model displays hallmarks of chronic allergic asthma mimicking human disease, e.g. airway hyperresponsiveness, a mixed neutrophilic and eosinophilic inflammatory response in the lung, proinflammatory cytokines and remodeling in lung tissue. This paper provides a relevant model for studying chronic allergic disease induced by a natural airway allergen. Thus, the model is suitable for testing novel strategies for cat allergy vaccination, for evaluating and developing new treatments of human disease.

LIST OF SCIENTIFIC PAPERS

- I. Konrad Wadén*, Natalija Polovic*, Jonas Binnmyr*, Carl Hamsten, Reidar Grönneberg, Carina Palmberg, Natalija Milcic-Matic, Tomas Bergman, Hans Grönlund, Marianne van Hage. *Shared first authorship
Dog saliva – an important source of dog allergens
Allergy, 2013, 68, 585–592
- II. Konrad Wadén, Danijela Apostolovic, Sara Sanchez-Vidaurre, Mirela Curin, Jeanette Grundström, Guro Gafvelin, Tanja Cirkovic Velickovic, Hans Grönlund, Wayne R Thomas, Rudolf Valenta, Carl Hamsten± and Marianne van Hage±.
±Shared last authorship
The novel cat lipocalin Fel d 7 and its cross-reactivity with the dog lipocalin Can f 1: Structures, epitopes, and allergenicity
SUBMITTED
- III. Konrad Wadén*, Anna Asarnoj*, Carl Hamsten, Niklas Andersson, Inger Kull, Christian Lupinek, Mirela Curin, Josep Anto, Jean Bousquet, Rudolf Valenta, Magnus Wickman‡, Marianne van Hage‡.*Shared first authorship, ‡Shared last authorship
Sensitization to Fel d 1 and Can f 1 in childhood predicts symptoms of cat and dog allergy in adolescence – a BAMSE/MeDALL study
MANUSCRIPT
- IV. Jeanette Grundström*, Tiiu Saarne*, Cecilia Kemi, Joshua A. Gregory, Konrad Wadén, Marina C. Pils, Mikael Adner, Guro Gafvelin‡, Marianne van Hage‡
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Development of a Mouse Model for Chronic Cat Allergen-Induced Asthma
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LIST OF ABBREVIATIONS

AHR	Airway hyperreactivity
Alum	Aluminium hydroxide
APC	Antigen presenting cell
ASIT	Allergen-specific immunotherapy
BAL(F)	Bronchoalvelar lavage (fluid)
BAMSE	Children Allergy/Asthma Milieu Stockholm Epidemiologic study
BAT	Basophil activation test
Can f	<i>Canis familiaris</i>
CD	Circular dichroism
CD	Cluster of differentiation
CDE	Cat dander extract
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
Fel d	<i>Felis domesticus</i>
HE	Hematoxilin/eosin
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cells
i.n.	Intranasal
MeDALL	Mechanisms for the Development of ALLergy
MHC	Major histocompatibility complex
MS	Mass spectrometry (LC-MS/MS, liquid chromatography tandem MS)
NK cell	Natural killer cell
OR	Odds ratio
OVA	Ovalbumin
PAMP	Pathogen-associated molecular pattern
PAS	Periodic acid-Schiff
P(N)PV	Positive (negative) predictive value
PRR	Pattern recognition receptor
r	Recombinant
RBL	Rat basophil leukemia
s.c.	Subcutaneous
SPT	Skin prick test
Th cell	T helper cell
TLR	Toll-like receptor
Treg	Regulatory T cell

1 THE IMMUNE SYSTEM

1.1 HISTORIC INTRODUCTION

The immune system has evolved to protect us from harmful foreign substances and has developed with us since we became multi-cellular organisms. The word ‘immunity’ comes from the Latin *immunis* and means ‘cannot be touched’. Immunity to disease was noted as early as 430 BC by Thucydides in Athens, where plague survivors resisted repeated infection after their initial exposure. However, it was not until the 1500th century that the Turks and Chinese, and finally Edward Jenner in 1798 in England, deliberately induced immunity to smallpox by inoculation with dried smallpox virus and cowpox virus, respectively. Since this advancement, medicine has become even more aware of the importance of our immune system.[1]

1.2 INNATE AND ADAPTIVE IMMUNITY

1.2.1 The Innate immune system

The immune system is comprised of two parts working together; the more primitive innate immunity and the later, evolved adaptive immunity. The innate immunity is encoded in our genes and specializes in protecting and quickly eliminating common pathogens by physical, chemical and molecular barriers. To our immediate help against pathogens, we have an outer barrier of epithelia on our skin and mucosal surfaces inside our body together with a protective bacterial layer, microbiome, on all surfaces including healthy bronchus. If a pathogen breaches the outer defense, our innate immunity forms a first line of defense with anti-microbial substances, phagocytosis, cellular responses, cytokines and the complement system that respond within seconds to hours. The response is mainly inflammatory, and inflammation is characterized by increased vascular permeability and blood flow leading to heat, pain, redness and swelling, or *calor, dolor, rubor* and *tumor* in Latin. This inflammation gives access to infiltrating immune cells to the attacked area. Key cells in the innate immunity are Natural killer (NK)-cells, Neutrophils, Macrophages (M ϕ) and Dendritic Cells (DCs). [1] A new and lesser explored group of innate immune cells are innate lymphoid cells (ILCs) that are divided in types similar to T-cell classification, described later. The most well-known ILCs is the NK-cell (ILC1), ILC2s are related to T helper type 2 cells and relevant for allergies [2, 3]. The innate immunity uses preformed receptors to quickly initiate a primary immune response when stimulated by their ligands. These are more primitive pattern recognition receptors (PRRs) that respond to damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). The PRRs, like the Toll-like receptors (TLRs), are germline encoded and inherited as an evolutionary protection against known pathogens [4]. However, the pathogens often mutate to adapt and escape our innate immune system. [5]

1.2.2 The Adaptive immune system

Through the parallel evolution with microorganisms we have developed a second immune defense, the adaptive immune system, with T-cells and B-cells and the ability to recognize almost any foreign antigen by having random antigen receptors. When lymphocytes are developed, B-cells in the bone marrow and T-cells in the thymus, combinations of hundreds of gene segments of variable regions of their antigen receptors are joined. This creates thousands of variations of the variable regions on the heavy and light chains of the receptor that is unique to every cell. The variation is further amplified by junctional diversity because nucleotides are added or subtracted in the gene segment-joining process. Hundreds of millions of combinations are created by this combinatorial diversity of the antigen receptor. Most B and T-cell receptors (BCRs and TCRs) will not recognize anything, but when a pathogen is recognized by the receptor these cells undergo selection and proliferation. However, before any pathogen or antigen (Ag) can be recognized they need to be presented to the receptors. The presentation bridges between the innate and the adaptive immunity. Macrophages, some B-cells and especially DCs are specialized to effectively monitor their surroundings by constantly engulfing, or phagocytizing, it and are termed “professional antigen presenting cells” (pAPCs). Many cell types, especially during inflammation, have the ability to function as APCs but are less effective. The antigens are taken up, processed enzymatically and presented by Major Histocompatibility Complex (MHC) class I and class II to T-cell receptors in the lymph nodes, which may start an adaptive immune response. [1] Pathogens if in the cell cytosol are processed and presented to on MHC class I on all nucleated cells initiating CD8⁺ cytotoxic T-cell response to kill infected or damaged cells. In the exogenous pathway, phagocytized pathogens are presented to CD4⁺ helper T -cells on MHC class II on pAPC only. The CD4⁺ -cells can then differentiate into many functions including effector T-cells, memory T-cells, or regulatory T-cells. [6]

1.3 HUMORAL AND CELLULAR IMMUNITY

1.3.1 Humoral immunity

Adaptive immunity is divided in humoral- and cellular immunity. Humoral immunity is antibody-based. Naïve B-cells undergo a selection process during development in the bone marrow (however their origin in birds, *bursa of Fabricius*, was discovered earlier and gave rise to the name “B”-cell) that leads to the expression of a single BCR, i.e. a membrane bound antigen-specific immunoglobulin.[1] Naïve B-cells have not found their specific Ag and have not been activated yet. Circulating in secondary lymphatic tissues (lymph nodes and spleen), they are activated upon encounter of antigens binding to a specific BCR and enter germinal centers where they undergo affinity maturation toward the Ag and class switch recombination (CSR) to an immunoglobulin (Ig) isotype (see below). Orchestrated by presentation of extra cellular antigen on MHC class II, CD4⁺ T-helper-cells activate B-cells via their BCRs which differentiate into either plasma cells that produce antigen specific

antibodies or into long-lived B-cells with specific BCRs recognizing the Ag and make our defense both faster and stronger at subsequent encounters. [7] This immunologic memory is the basis of vaccination in T-cell dependent B-cell response. B-cell receptors are membrane bound immunoglobulins (Ig) of five classes or isotypes; IgA, IgD, IgE, IgG and IgM. There are antigens that can evoke T-cell independent B-cell response but only IgM and no memory response. Immunoglobulins are glycoproteins that consist of two light chains joined by disulfide bonds with two heavy chains forming a Y-shape. Variable regions on the tips of the heavy and light chains form the antigen-binding site and bind the antigen, while the constant region of the heavy chain defines the class, which in turn determines the effector mechanism of the different Igs. All mature B-cells start by expressing IgM, but binding of the antigen together with co-activation from T-cells activates the B-cells to class switching into more specialized IgA, IgG or IgE antibodies better suited to fight particular antigens. Secreted Igs, or antibodies (Ab), are central in immune recognition and in mounting immune responses to pathogens.[1]

1.3.2 Cellular immunity

The other part of adaptive immunity is the cell-mediated immunity that does not rely on antibodies, but on T-cells and the induction of phagocytes and cytokines. It includes cytotoxic CD8⁺ T-cells (T_{cyt}-cells) and CD4⁺ helper 1 T-cells (Th1) that are able to kill cells infected by intracellular viruses or bacteria. Specific CD8⁺ T cell responses are primed by APCs in secondary lymphatic tissues. This activation is orchestrated by MHC class I presenting intracellular antigen to naïve CD8⁺ cytotoxic T-cells that kill cells recognized by their TCRs. CD4⁺ Th1-cells are activated via MHC class II, leading to IFN γ production, which in turn activates macrophages and increases their microbicidal activity. One important part of the immune system and cell mediated killing in particular is tight control. Mechanisms like feedback loops and homeostasis usually protect the body from our immune system going out of control. [3]

1.3.3 Th1/Th2 paradigm

Since the original presentation of the Th1/Th2 paradigm in 1986 [8] the helper T-cells have been divided into, and seen as a balance and competition between, two Th subsets. These Th subsets, Th1 and Th2, are focused on protecting the host against intracellular microbes and extracellular helminths etc., respectively. Th1 immunity consists of cells with the lineage marker Tbet that typically produce the cytokine IFN γ , while Th2 immunity consists of cells expressing the lineage marker GATA-3 that typically produce IL-4, IL-5 and IL-13, which induce anti-body production. Lately, it has become more and more clear that this classification is oversimplified. More subclasses have been identified and the plasticity among Th subsets is greater than it was first thought to be. The Th17 subset is a third distinct but plastic lineage with ROR γ t positive cells that produce the signature cytokine IL-17 and are specialized in fighting extracellular bacteria and fungi.[3, 9] Other Th-cell subsets that have been established are Th9[10, 11] and Th22[12], producing the signature cytokines IL-9 and

IL-22, respectively, and expressing the transcription factors IRF4/PU.1 (Th9) and the aryl hydrocarbon receptor (AhR) (Th22).[3]

1.3.4 T-regulatory cells

Tolerance, as mentioned earlier, is a key concept in the immune system. T-regulatory (Treg) cells are commonly defined as CD25^{high}, CD4 and Foxp3 positive and CD127 negative T-cells [13]. These mainly refer to thymus-derived, or natural, Treg cells. Peripheral induced, or adaptive, Treg cells may be Foxp3 positive or negative. Treg cells have the ability to end an inflammatory state to keep homeostasis, influence (i.e. dampen) other cells and maintain peripheral tolerance. Tregs regulate other immune cells by e.g. producing inhibitory cytokines like IL-35, IL-10 and TGF- β , or by cell contact through e.g. inhibitory surface molecules like CTLA-4 that blocks CD80 and CD86 co-stimulatory molecules.[14]

1.4 ALLERGY

1.4.1 History of allergy

Allergic diseases have been described already in 900 AD when the Persian physician Rhazi described seasonal rhinitis [15], but it took a long time before the modern nomenclature was coined. In 1902, the French physiologists Richet and Portier observed that dogs experience immediate anaphylaxis and even death when exposed to a second dose of a small protein toxin of sea anemones. Instead of giving protection, or *phylaxis*, as expected, the prior injections made the immune system hypersensitized.[16] The term allergy was finally introduced in 1906 by the Austrian pediatrician Pirquet, and refers to *allos* 'other' and *ergon* 'work'. [17] IgE, which is central in allergic disease, was first described as 'reagin' in 1922 and characterized as IgE in 1966. [18, 19]

1.4.2 Types of allergy

The prevalence of allergy is about 30%. [20-24] Typical allergy symptoms are runny nose, red eyes or skin reactions like itching, eczema, urticaria, as well as more severe symptoms like asthma and anaphylactic reactions. The nomenclature for allergic diseases has been unified to allow scientists and different medical disciplines, as well as the public, to correctly address allergic and hyper-reactivity problems.[25]

Allergy is a hypersensitivity initiated by an immunologic mechanism. There are non-allergic hypersensitivities and allergic hypersensitivities; the latter is referred to as allergy. Allergic hypersensitivity is in turn divided into IgE-mediated allergy and non IgE-mediated allergy (Fig. 1). Atopy is connected to allergy and refers to individuals who are genetically predisposed to produce IgE antibodies in serum to normally harmless environmental agents, allergens. The definition of atopy is: "A personal or familial tendency to produce IgE antibodies in response to low doses of allergens, usually proteins, and to develop typical symptoms such as asthma, rhinoconjunctivitis, or eczema/dermatitis." [25, 26] IgE-mediated

allergy is the most common type of allergic hypersensitivity and the majority of patients are atopic. There are however some exceptions; for example, wasp allergy is not linked to atopy. Allergic contact dermatitis is an example of non IgE mediated hypersensitivity.[25, 27]

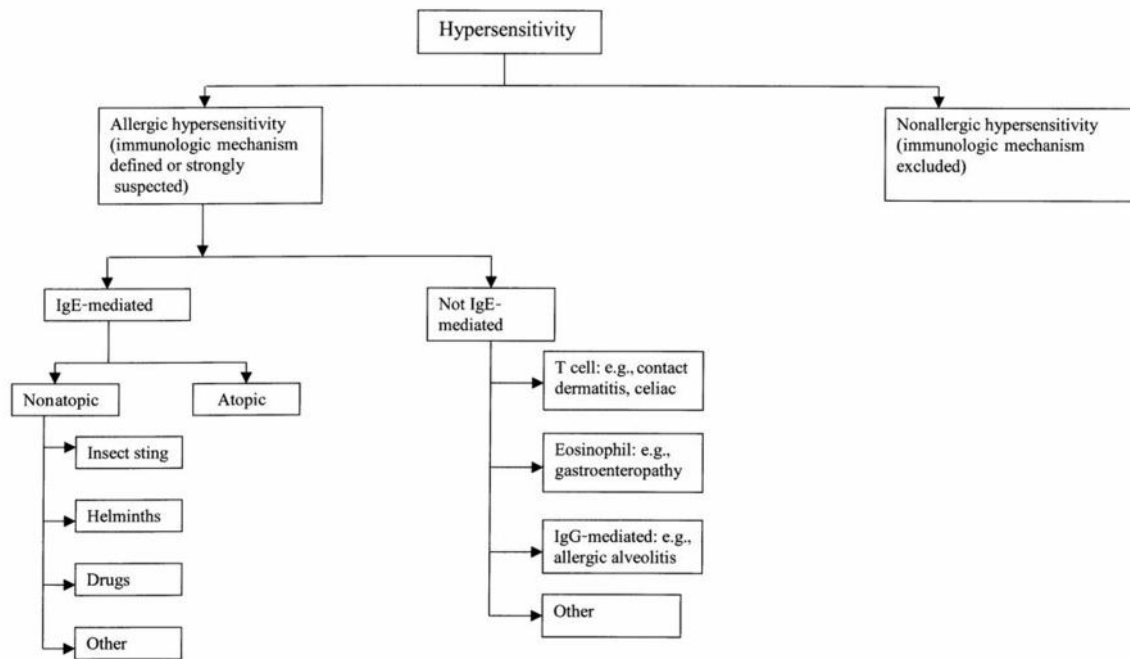


Figure 1. Classification of hypersensitivities and allergies. Re-Printed from and with curtesy to S.G.O. Johansson.[25]

1.4.3 Allergic sensitization

The up-to-date explanation of allergy is that when certain individuals that are most often atopic encounter an allergen for the first time, various parts of their immune system start to overreact. Allergic reactions are overly-powerful, or faulty, responses to normally harmless environmental antigens, or allergens. It is not known why some proteins become allergens. PRRs like TLRs on APCs have been shown to interact with some allergens, possibly mistaking them for pathogens. APCs migrate and present the allergen to T cells in the lymph nodes.[1] The immune response depends on the location, the cytokine milieu around the cell and the co-stimulatory signals. The IgE antibody production in allergy requires Th2 cells and is inhibited by Th1 cells and INF .[5, 28] Also the route and dose to the allergen exposure have importance. A low dose favors an allergic Th2 response and many common allergens are inhaled and delivered to the lungs in low dose. [1, 29] DC mediated priming of naïve Th cells into Th2 are very important in deciding over induction of Th2-cells and production of proinflammatory cytokines in the induction of allergic reactions.[30] Th2 cells expand and produce cytokines like IL-4, IL-5 and IL-13 that further promotes allergic inflammation, Th2 expansion, B cell class switch to production of IgE, recruitment, differentiation and

maturation of basophils, mast cells and eosinophils. In this sensitization phase, IgE antibodies are secreted into circulation and bind to the α -chain of the high-affinity receptors for IgE (i.e. Fc ϵ RI) on the surface of mast cells and basophils. This results in these cells and the persons becoming sensitized to that allergen.[1] In healthy individuals, Tregs help to suppress reactions towards allergens, whereas allergic individuals have been shown to have an impaired Treg response to allergens due to a Th2 cytokine milieu that inhibits Treg development.[31]

1.4.4 Early phase reaction

In the effector phase of allergy, a new encounter with the allergen causes cross-linking of IgE antibodies on sensitized mast cells and basophils. These cells are activated and within a few minutes degranulate to release preformed and newly generated inflammatory mediators, mainly histamine, heparin and proteases. Preformed pro-inflammatory cytokines like TNF α , IL-2, IL-4, IL-5 and IL-13, together with chemokines, are released and stimulate the production of more cytokines and chemokines while the degranulated mast cells and basophils start producing leukotrienes and prostaglandins. The inflammatory response is characterized by increased vascular permeability and blood flow, which leads to heat, redness and swelling. This causes bronchoconstriction, airway mucus secretion, itching, rashes and possible systemic anaphylaxis vasodilation.[1] Anaphylaxis or anaphylactic shock is not the most common allergic symptom but is the most severe. Systemic effects give an acute fall in blood pressure followed by contraction of smooth muscles with stress on the heart and lungs that can be fatal within minutes of contact with the allergen. [32, 33]

1.4.5 Late phase reaction

Several hours after allergen exposure, the activation of allergen-specific T-cells can in some patients induce a late phase allergic reaction with edema of the skin (after a skin prick test), blockage of the nose, and narrowing of the airways and mucus hypersecretion. The reactions are induced by cytokine-mediated recruitment and activation of large numbers of Th2 cells, neutrophils, eosinophils, basophils and resident mast cells.[1] Anaphylaxis can also be delayed and occur during the late phase.[33]

1.4.6 Chronic phase

If the allergic inflammation is sustained over an extended period of time, extracellular matrix and structural cells can be affected to the point that chronic inflammation can lead to tissue remodeling and fibrosis, coupled with the development of allergic asthma. [33] The sustained eosinophilic Th2-polarized inflammatory state with excess of e.g. IL-4 and IL-5 starts a process where excess of extra cellular matrix production and fibrous connective tissue forms a scarred area and fibrosis permanently damage to the lung tissue.[28, 34] This damage leads to progression of asthma and decrease of flexibility of the lung with asthma symptoms as result.

1.4.7 Etiology

1.4.7.1 Genetics and epigenetics

About half the western urban population is atopic and the majority of people with allergy are atopic. [35] Atopic families and individuals have a concentration of genes or gene variants in areas related to endogenous induction of inflammation associated with allergic asthma.

Allergic rhinitis, grass sensitization and cat allergy have been coupled with other genes typically related to Interleukins and MHC/HLA. However, as in all complex diseases, the genetic contribution is only one part of the reason why symptoms occur; it is possible to develop allergies without being atopic. Another part is epigenetics, first used by Conrad Hal Waddington in 1940 for the layer above the genes regulating somatic gene expression modifications.[36] Environmental exposures can influence the epigenome effecting e.g. pro-inflammatory cytokines related to allergic disease and the changes can be inherited.[2, 37] However, inherited factors alone cannot explain the etiology of allergic disease.

1.4.7.2 Hygiene hypothesis

There has been a marked increase in allergic diseases in children and young adults [38] that has reached epidemic proportions worldwide, and the incidence of these diseases is continuing to increase in association with a western metropolitan lifestyle. [35, 39, 40] Environmental exposure to potential allergens early in life or during pregnancy probably has a key role in the development of allergy[41, 42]. Increased prosperity, health, new diets and environment are all hypothesized to contribute to the increasing number of allergic diseases due to under-stimulation of the immune system, making it over-sensitive. This explanation is referred to as the 'Hygiene hypothesis'. Countries now experiencing the development seen in the western world a few decades ago are also accompanied by increases in allergic diseases, e.g. the Baltic countries and, more recently, China. Now more and more data supports the hygiene hypothesis. [43-45]

1.5 ALLERGENS

1.5.1 Allergen families

There are major and minor allergens. The definition of a major allergen is that IgE sensitization to the allergen is found in over 50% of the patients allergic to the allergen source it originates from. Minor allergens have a prevalence of sensitization of less than 50%. [46, 47] Allergens are named after the Latin names of their origin and are, as a rule, numbered according to the order they were discovered and reported to WHO/ International Union of Immunological Societies (IUIS).[46, 48, 49] Allergens are proteins or glycoproteins, typically globular proteins and often with enzymatic activity. The enzymatic activity can be protease activity, as in the major mite (*Dermatophagoides pteronyssinus*) allergen Der p 1, by which e.g. tight junctions are digested facilitating the allergen access across epithelial barriers.[50-52] Other allergens have enzymatic activity that stimulates other paths. Many allergens are

similar to their human counterpart proteins, while they need to be different enough to be allergenic. With respect to mammalian allergens, lipocalins is the largest allergen family, and more than 50% of the total allergens identified from furry animals belong to this protein family.[53] They are found in many body fluids and secretions including urine, tears, saliva, serum and dander. All lipocalins share a conserved structure and folding pattern, even if they often only share some sequence identity.[54-56] The only other main pet allergens not belonging to the lipocalin family are the major cat (*Felis domesticus*) allergen Fel d 1, a salivary secretoglobulin[57, 58], and the major dog (*Canis familiaris*) allergen Can f 5, a urinary kallikrein[59]. Serum albumin is another common allergen family that is present in most pets.[40, 60-65]

1.5.2 Allergen Extracts

Allergen extracts are mixtures of allergens and non-allergenic material, e.g. enzymes and other proteins extracted from an allergen source. Pet allergen extracts are typically prepared by extracting proteins from hair and skin scrape i.e. dander. Saliva, and urine to some extent, are transferred to fur and can often be found in these kinds of extracts. Some allergens may be poorly represented in crude allergen extracts among non-allergenic molecules or unproportional amounts of other allergens from the same source.[66] Smaller animals such as mites or ticks, or food and plant allergens that are often present in the allergen source, may be present in the extract and pet extracts have been shown to cause false positive results, due to contamination.[67-69] These impurities can even lead to new sensitizations during treatments with extracts.[70] The advantage of extracts is that they resemble the allergen sources in composition and are similar to how we are exposed to the allergens in real life. Unfortunately, even with attempts to standardize production the content and the quality of allergen extracts have shown great variability.[66]

1.5.3 Cat allergens

Allergy to cats (*Felis domesticus*) is the second most common indoor allergy in the western world for both children and adults. The major cat allergen is Fel d 1, which is a salivary uteroglobin protein,[58, 71] is a very dominant cat allergen. Up to 95% of all cat-allergic patients have IgE against Fel d 1.[72-74] Fel d 2 is a serum albumin, Fel d 3 a cystatin and Fel d 4 a lipocalin. Fel d 5 is cat IgA and Fel d 6 cat IgM, which contains the carbohydrate galactose-1,3-galactose (alpha-Gal)[75]. The newly identified Fel d 7 and Fel d 8 belong to the lipocalin and salivary latherin family, respectively.[76, 77] The prevalence of IgE reactivity among patients allergic to cat for Fel d 2 is around 20%, Fel d 3: 10%, Fel d 4: 60%, Fel d 5: 40%, Fel d 6: NA, Fel d 7 38% and Fel d 8: 20%, summarized in [53].

1.5.4 Dog allergens

The major dog (*Canis familiaris*) allergen Can f 1 together with Can f 2, Can f 4 and Can f 6 belong to the lipocalin family.[54, 78-81] Can f 3 is serum albumin[82] and Can f 5 is prostatic kallikrein. Can f 5 derived from dog urine was identified as a major allergen in 2009 and reported to be recognized by up to 70% of dog-sensitized patients.[59] The importance of Can f 5 as a diagnostic marker for dog allergy is however questionable. The lipocalin Can f 6 is the most recently discovered dog allergen.[59, 78] The prevalence of IgE reactivity among patients allergic to dog for Can f 1 is around 50%, Can f 2: 25%, Can f 3: 20%, Can f 4: 20%, and Can f 6: 40%, summarized in [53].

1.5.5 Cross-reactive allergens

Cross-reactivity to allergens, i.e. IgE-binding to similar epitopes in different protein or to homologous proteins, is often found among lipocalins and serum albumins in pets. The fact that allergens from different furry animals often belong to the same allergen families has raised questions about cross reactivity. Investigations have determined that these lipocalins and serum albumins are responsible for most of the cross reactivity that is seen.[54, 78, 83-86] The largest possibility to find cross activity is when the allergens have more than 60% sequence identity.[78]

Epitope spreading is related to the progression of allergic disease and, much like cross reactivity, is caused by IgE receptor presenting an allergen with a shared epitope to which the allergic individual is already sensitized. This process can lead to sensitization to new adjacent epitopes and new allergens if some epitopes are shared between the different allergens. [87, 88]

1.5.6 Recombinant allergens

Advances in molecular biology during the recent decades have enabled the generation of recombinant allergens, with well-defined molecular, immunological and biological characteristics.[89-91] The first allergen to be cloned was Der p 1 in 1988.[92-94] This opens up possibilities to produce standardized products of reliable purity. Genes of the allergens are cloned into expression vectors and transformed in to *Escherichia. Coli* production systems like e.g. BL-21. Recombinant (r) proteins can be produced consistently and with very high purity, and are therefore very attractive for diagnostics as well as treatments and can help in increasing specificity.[83, 95] Recently, allergenic molecules from the most important allergen sources have become available as recombinant proteins.

1.6 ALLERGY TREATMENT

1.6.1 Symptomatic treatment

The first approach to relieve allergic symptoms is avoidance of the allergen source, but some allergens are hard to avoid. As a next step, a variety of medications are utilized for

symptomatic therapy, e.g. anti-histamines, anti-leukotrienes and inhaled steroids.[1] These kinds of treatments only give short term relief from the symptoms and lifelong treatment is often needed. Acute anaphylaxis is treated by intramuscular adrenalin (epinephrine) injections that relax respiratory smooth muscles and acts on the heart.[32] A drug given exclusively in special cases such as severe allergic asthma with high IgE levels is the humanized monoclonal antibody Omalizumab (Xolair). Its specificity is against circulating IgE and works by blocking the IgE from binding to the IgE receptors on the mastcells and basophils. [96] However, a lifelong treatment regime is not ideal.

1.6.2 Curative treatment

The word vaccination springs from ‘vacca,’ Latin for cow, and originates from the phenomena of transferred immunity. It was described by E. A. Jenner in “An Inquiry into the Cause and Effects of the Variolae Vaccinae” in 1798.[97] Allergen Specific Immunotherapy (ASIT), lately termed allergy vaccination, is the only treatment that can cure allergic disease, and it has been performed for over 100 years[98]. In 1911, the British scientist Leonard Noon treated grass pollen allergic patients with grass extract. He injected patients with the extract over several weeks in increasing amounts and successfully alleviated hay fever symptoms.[99] ASIT has been proven to be a clinically effective treatment for allergic disease. It is the only disease-modifying therapy that can reduce allergic inflammation and prevent the development of chronic disease.[100] Typically an adjuvant is used in ASIT to increase to effect of ASIT. Alum is currently the adjuvant used in subcutaneous ASIT (SCIT), but our research group and others has also evaluated other adjuvants and approaches for better and safer ASIT. Alum mainly promotes Th2 immune responses [101], which is not ideal in an allergy context, and its main function in allergy vaccines is to prevent systemic spreading of the allergen. Moreover, a common adverse side effect to SCIT is granuloma formation at the injection site, caused by alum [102]. The aim with an adjuvant in ASIT is to modify the allergen-specific immune response to tolerate an allergen by changing the immune response to the allergen from allergic Th2 to tolerogenic Treg or Th1 and by induction of blocking IgG-antibodies that hinder allergen binding to IgE.[103-105] Most commonly, ASIT is performed by subcutaneous injections of the allergen extract (SCIT). Newer safer alternatives are sublingual immunotherapy (SLIT)[106, 107] and intralymphatic immunotherapy (ILIT)[108] where tablet/drops are given orally or injections in to lymph nodes, respectively. In the latter, sine it is given directly into the lymph node, only a very small dose needs to be given reducing the risk of side effects. The oral route is convenient since patients can administer the tablets/drops themselves, but has shown to be less efficient compared to SCIT.

However, traditional ASIT protocols similar to the SCIT original protocol by Noon are long, up to five years, as well as time- and resource-consuming and are associated with risks of both early and late adverse side effects.[109] In order to make the treatment attractive for more patients that would benefit from ASIT, new safer and shorter treatment strategies with

fewer injections and new administration routes are currently under development and assessment. [95, 110-117] We implement the knowledge into a future treatment of allergy via mouse models. Most mouse model are acute, meaning that they model the acute phase of allergic disease but a few, including our, are chronic models that also mirror the chronic stages of human allergic disease.

2 THESIS AIMS

The overall aim of this thesis is to identify and characterize new pet allergens and to use the findings to improve diagnostics and the prediction of pet allergy. And furthermore, to provide a relevant platform, a mouse model reflecting chronic asthma, for the development of novel treatment strategies for cat allergy. The specific objectives of the four papers are:

Specific aims:

Paper I: To evaluate dog saliva as a source of new allergen molecules for improved diagnostics of allergy to dog.

Paper II: To investigate the prevalence of sensitization to the novel allergen Fel d 7 in a European cat-sensitized population and elucidate its allergenicity and cross-reactivity with the dog allergen Can f 1 on an epitope level.

Paper III: To investigate the association between sensitization patterns to cat and dog allergen molecules during childhood and symptoms to these furry animals up to 16 years of age.

Paper IV: To establish a relevant model for cat allergen-induced asthma in mice, exhibiting features of human chronic disease.

3 METHODS

The methods used in the different papers included in this thesis are briefly described in this section with reference to the papers where they are applied. Each paper contains a section with detailed descriptions of the material and methods.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to determine the content of e.g. antibodies or cytokines in fluids, e.g. sera or BAL. Different types of ELISAs were used where an antigen or an antibody was coated. Inhibition ELISAs were conducted with serial dilutions of inhibitors mixed with serum prior to the ELISA. A reference standard for e.g. IgE or IgG was used when quantifying absolute levels. ELISA was used in paper I, II and IV.

ImmunoCAP

A routine diagnostic method, ImmunoCAP, was used for determining allergen-specific IgE (ImmunoCAP System; Phadia/Thermo Fisher, Uppsala, Sweden) in which an allergen molecule or allergen extract is coupled to a solid phase. The allergen-specific IgE levels are given in kUA/L. This method was used in paper I, II and IV.

Luminex

Luminex is a technique reminiscent of ELISA but that simultaneously measures and quantifies multiple protein targets in a single fluid, e.g. serum, sample. This method was used in paper IV.

MeDALL chip

The MeDALL (Mechanisms for the Development of ALLergy) microarray is based on the, ISAC technology. The values are given in ISAC Units (ISU-E). This method was used in paper III.

Protein production and purification

Genes of allergens of interest; Fel d 1, Fel d 7 and Can f 1, were cloned into expression plasmids and transformed into Escherichia. Coli production systems. Bacterial pellets were solubilized and purified by e.g. His-tag specificity on immobilized metal chelate affinity chromatography (IMAC) and size. These methods were used in paper I, II and IV.

Biochemical characterization

Allergen proteins were analyzed by SDS-PAGE to verify molecular size and purity. Some gels were used for immunoblotting or 2D-PAGE before mass spectrometry. These methods were used in paper I and II.

Immunoblot

Proteins of interest were transferred from SDS-PAGE gels to membranes and detected using specific antibodies and a secondary enzyme-conjugated detecting antibody. This method was used in paper I

Mass spectrometry

Protein extracts from different sources were separated by 2D-PAGE. Protein dots of interest were excited, trypsin digested and analyzed by LC-MS/MS (Liquid chromatography– Tandem mass

spectrometry or) for identification. This method was used in paper II.

Mouse model

Different protocols were used in which mice were presensitized subcutaneously with alum-adsorbed recombinant cat allergen Fel d 1, followed by intranasal challenges with cat dander extract spiked with recombinant Fel d 1 or PBS. For reference, mice were presensitized and challenged with ovalbumin following the same protocol. This method was used in paper IV.

Airway hyperresponsiveness measurements

Airway hyperresponsiveness (AHR) was analyzed by a direct invasive method, using a small animal ventilator, FlexiVent. The airway response to increasing doses of metacholine is recorded using computerized measurements of pressure and air flow. From these data lung physiological parameters such as resistance and elastance can be calculated. This method was used in paper IV.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed directly after AHR measurement to assess the inflammatory response to AW challenge. Lungs of mice were flushed with PBS to collect BAL cells and fluid (BALF) for differential cell counting and cytokine measurements, respectively. This method was used in paper IV.

Lung remodeling analyses

At the end of the mouse protocol, lungs were subjected to histological analyses of inflammation and tissue remodeling. Paraffin-embedded lung sections were stained by hematoxylin/eosin (HE) and periodic acid-Schiff/Alcian blue reaction (PAS) for detection of infiltrating cells and mucus and with Masson's trichrome for analysis of connective tissue. This method was used in paper IV.

Quantitative PCR

At the end of the mouse protocol, lung tissue cDNA was amplified from mRNA expression of particular genes e.g. Th2 cytokines (IL-4 and IL-5 mRNA expression) analyzed and quantified in comparison to a reference gene. This method was used in paper IV.

Basophil activation test

In the basophil activation test (BAT), basophils were tested for reactivity and sensitivity to different stimulants. Whole blood samples from allergic patients were incubated with serial dilutions of allergen and then stained for the basophil marker CD203c and the degranulation marker CD63. This method was used in paper I and II.

Rat basophil leukemia assay

Rat basophil leukemia (RBL) cells transfected with the human high-affinity IgE receptor Fc RI were incubated with sera from sensitized patients and stimulated with different concentration of allergens. Release of α -hexosaminidase from RBL cell was measured in ELISA. This method was used in paper III.

Circular dichroism spectroscopy

Far and near UV circular dichroism (CD)-spectra were performed to obtain information on

secondary and tertiary protein structures for protein characterization and to compare structures between proteins. This method was used in paper II.

3D modeling

Sequences of allergen molecules of interest were compared using SWISS-MODEL to predict the 3D structure. This method was used in paper II.

Rabbit immunization

Peptide-specific IgG antibodies were obtained by immunizing rabbits three times with Can f 1-derived synthetic peptides. This method was used in paper III.

Statistical analyses

Association between sensitization patterns to allergen molecules and symptoms were analyzed for e.g. Odds Ratios (OR) and Positive/Negative predictive values (PPV/NPV). Results from treatment groups from the mouse asthma model protocol were compared by Mann-Whitney U-test. These methods were used in paper III and IV, respectively. In addition Spearman Correlation test (in paper I,II) and linear regression as well as t-tests (in paper III) were used.

Ethical statement

All human studies and animal experiments conducted in paper I, II, III and IV were approved by the local ethics committees. Written consent was obtained from all patients recruited for the basophil experiments.

4 RESULTS AND DISCUSSION

4.1 IDENTIFICATION OF DOG SALIVA ALLERGENS AND THEIR IMPROVEMENT OF DOG ALLERGY DIAGNOSTICS [1]

In this paper, we studied dog saliva as a source of allergens for improved diagnosis of allergy to dog. We compared dog saliva and dog dander extracts in western blot and observed that the dog-allergic patients had IgE specific to many salivary proteins. There was a greater abundance and diversity of IgE-binding proteins in dog saliva compared to dog dander extract (Fig. 2). In dog dander extract, most of the patients recognized protein bands at positions corresponding to sizes of known dog allergens. Contrastingly, at least 12 IgE-binding proteins were detected in dog saliva and several of those were of molecular weight sizes not recognized in the dog dander extract (Fig. 2).

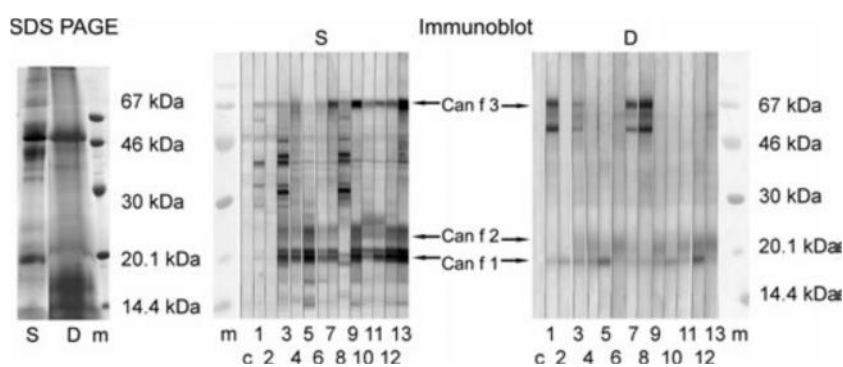


Figure 2 SDS–PAGE and immunoblot analyses of dog saliva (S) pool (n = 14) and dog dander (D) extract (Allergon). Immunoblot was developed with single dog-allergic patient's sera. m, Molecular weight markers; c, control (buffer); lanes 1–13, patient sera.

We looked further into the IgE binding in dog dander and dog saliva by a 2D-PAGE by using a pool of dog allergic patient's sera (n=13). The seven most prominent IgE binding spots detected among dander (five) and saliva (two) samples were cut out, trypsin in-gel digested, analyzed and identified by LC-MS/MS sequencing of peptides and database searches. From the saliva sample five spots were analyzed, and Can f 1, 2, 3 and 6 peptides were identified in one or more spots. We also found four new saliva allergen candidates in the dog saliva. These were two BPI fold proteins and dog IgA heavy chain constant region. From the dog dander extract two spots were analyzed, and Can f 1, 2, 3, 4 as well as Can f 6 together with BPIFA1 peptides were identified in one or both spots. Verifying the known allergens strengthen the data on the new allergen candidates.

The majority of dog dander positive patient sera (44 of 59) were also IgE positive to dog saliva in ELISA. More than one-third of these patients (23/59; 39%) had a higher IgE reactivity to saliva than to dander. Among 55 dog dander negative patients with symptoms to dog, 20% were IgE positive to dog saliva. As diagnosis of dog allergy in daily practice relies on the clinical history of the patient together with diagnostics based on dog dander extract,

our results have important clinical implications. We observed that the correlation between IgE responses to saliva and dander in ELISA was $r^2 = 0.48$ ($P < 0.0001$) (Fig. 3), reflecting that the two allergen sources are related, but more interestingly, that they also differ in their allergen content. This gives the potential to utilize dog saliva for future improved diagnostics of dog allergy. One approach would be to spike dog dander extract with dog saliva. Since saliva is full of degradation enzymes and unstable, another option would be to apply the newly identified dog allergens from saliva in component resolved diagnostic to get data on the individual allergen molecules.

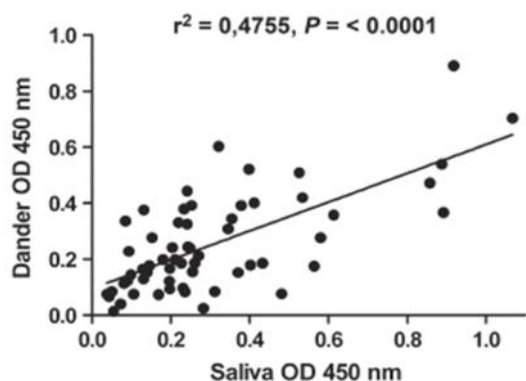


Figure 3 Correlation between IgE reactivity to dog dander (y-axis) and dog saliva (x-axis) ($n = 59$); OD – optical density, r^2 – correlation factor.

The allergenic activity of saliva was confirmed by basophil degranulation in three patients, emphasizing that saliva is an allergen source. Interestingly, one of these patients was low or non-responding to dog dander extract. Dog saliva gave rise to similar or higher basophil activation than dog dander in all three patients. The results strengthen the importance of improving dog dander based diagnostics by dog saliva. Other allergen sources, such as urine, might also hold undiscovered dog allergens. A recently discovered urinary major dog allergen is Can f 5. However, Can f 5 is deposited on male dog hair and dander [59].

We analyzed saliva from different dog breeds and noted that there are great variations in the IgE-binding profile where some had fewer IgE binding bands and the amount of different proteins varied. Overall, a greater abundance and diversity of IgE-binding proteins was found in dog saliva compared to dog dander extract. We showed that there are many false negative results in current dog allergy. About one-fifth of patients with symptoms to dog, but lacking IgE antibodies to dog dander, were IgE positive to saliva.

Taken together, we show that there are at least 12 protein bands in dog saliva that are recognized by IgE antibodies from dog-allergic patients. Furthermore, we demonstrate that about one-fifth of patients with symptoms to dog, but lacking IgE antibodies to dog dander, were IgE positive to saliva. Thus dog saliva was shown to be a significant allergen source that should be taken into account for improved diagnostics of dog allergy. Combining dog dander and dog saliva or spiking dog dander extract with dog saliva would be beneficial for developing enhanced dog allergy diagnostics.

4.2 ALLERGENIC CHARACTERIZATION OF THE CROSS-REACTIVE LIPOCALIN CAT ALLERGEN FEL D 7 [II]

In this paper, we studied the newly discovered cat lipocalin allergen Fel d 7 and report that it is an important allergen in a Swedish cat sensitized population. As the primary structure of Fel d 7 has a high sequence similarity and identity (63%) with Can f 1 we also investigated its cross-reactivity with Can f 1. In SDS-PAGE we noted that both proteins appeared mainly as monomers in line with earlier reports. The near and far UV circular dichroism (CD)-spectra of rFel d 7 and rCan f 1 revealed similar secondary structure and tertiary structures.

More than a third of the Swedish cat dander-sensitized patients (37/94 individuals) were IgE positive to rFel d 7. The figure is similar to the one reported among cat allergic patients from Australia. The result shows that the newly identified lipocalin Fel d 7 is an important allergen in a Swedish cat sensitized population in addition to the very dominating major cat allergen Fel d 1. The Fel d 7-positive sera were also analyzed for IgE to rCan f 1 and the majority, 34 (89.2%), had IgE reactivity to rCan f 1 as well. We found a modest correlation ($r = 0.58$, $P < 0.001$) between the IgE levels to Fel d 7 and Can f 1, indicating that there is IgE cross-reactivity between these two lipocalins.

Even though Fel d 7 has been accepted as an allergen by the WHO/ International Union of Immunological Societies (IUIS) allergen nomenclature committee since 2011[76], its biological activity has so far not been elucidated. We investigated the allergenic activity of rFel d 7 by basophil activation test (BAT) using blood from cat allergic patients who were IgE positive to Fel d 7 and found that Fel d 7 was able to stimulate basophil degranulation. Thus, Fel d 7 is a biological active allergen. To further strengthen our finding we looked into the biological activity of Fel d 7 in relation to Can f 1 by analyzing sera from 11 patients IgE-positive to both rFel d 7 and rCan f 1 in a rat basophil leukemia (RBL) assay. We noted that the allergens induced a varying degree of degranulation that was independent of the allergen-specific IgE levels. We further explored the allergenic cross-reactivity between Fel d 7 and to Can f 1 by inhibition ELISA using sera sensitized to rFel d 7 and rCan f 1. We found that the degree of cross-reactivity varied between patients. Can f 1 was able to completely inhibit the IgE binding to rFel d 7 in two out of five sera, whereas rFel d 7 was able to inhibit the binding to rCan f 1 to 70% in two sera. The other sera showed lower degree or no inhibition. The results indicate that Fel d 7 has epitopes in common with the major dog lipocalin allergen Can f 1 which contributes to co-sensitization observed in patients with allergy to cat and dog.

We then aimed to identify the cross-reactive epitopes on Fel d 7 and Can f 1. Sera from rabbits immunized with five overlapping Can f 1 peptides spanning the Can f 1 sequence were used. We observed that peptides covering the C- and N-terminal epitopes gave rise to a stronger binding to rFel d 7 than the other antisera. Based on the results we created a 3D model, SWISS-MODEL, on the known model of human tear lipocalin/von Ebners gland protein (VEGP). Figure 4 shows that the main epitopes shared between Fel d 7 and Can f 1 are located at the C- and N-terminus (highlighted) which is consistent with our results from the Can f 1 peptide antisera (Fig. 4B). As Fel d 1 has shown not to account for all IgE

binding to cat extract, a larger cat allergen panel is needed in diagnostics. With respect to Fel d 7, we have shown that it is an important allergen in 39% of cat sensitized patients, and contributes to the co-sensitization noted between cat and dog allergic patients.

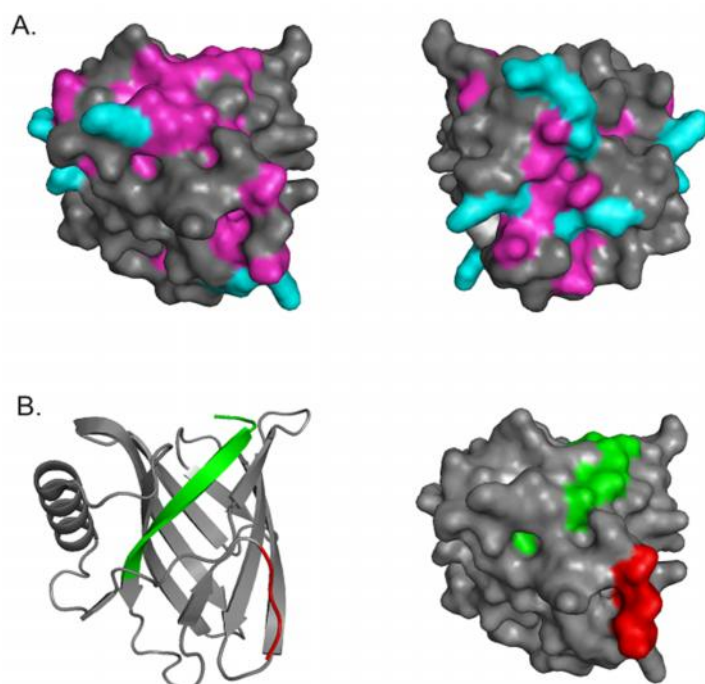


Figure 4 Cross-reactive epitopes on Fel d 7 and Can f 1.

(A) 3D model of cross-reactive sequences, pink color corresponds to sequences present in both pet lipocalins and the tear human lipocalin. Blue color is sequences present only in rFel d 7 and rCan f 1. (B) Highlighted N- (Green) and C-terminal (Red) ends.

4.3 PREDICTION OF PET ALLERGY IN ADOLESCENCE BY SENSITIZATION PATTERNS TO CAT AND DOG ALLERGEN MOLECULES IN CHILDHOOD [III]

We investigated sensitization to individual cat and dog allergen molecules in childhood through adolescence using the BAMSE (Barn/Children Allergy/Asthma Milieu Stockholm Epidemiologic study) birth cohort to identify risk markers for allergic symptoms to cat and dog up to age 16. Sera and questionnaire data from 779 randomly collected children at 4, 8 and 16 years were used. IgE reactivity to cat and dog allergen molecules were analyzed with the MeDALL (Mechanisms for the Development of ALLergy) chip. Allergy was defined as reported rhinitis, conjunctivitis or asthma at exposure to cat or dog.

We first investigated the prevalence of IgE sensitization to cat and dog extract (ImmunoCAP) and found that it increased over time, from 6.8% at 4 years to 19.8% at 16 years for cat and from 5.1% to 22.9% for dog. Likewise, allergic symptoms to cat and dog increased during the same period from 4.8% to 11.2% for cat and from 3.1% to 5.5% for dog. We also found that the prevalence of IgE to any of the cat or dog allergen molecules increased and reached nearly 15% and 22% at age 16 for any of dog or cat allergen molecules, respectively. Interestingly, sensitization to Can f 5 (1.9-12.6% at 4-16 yrs) was more common than to Can f 1 (1.9-5.5% at 4-16 yrs), but Can f 1 induced the highest IgE levels.

We next investigated IgE reactivity cross-sectionally and found that the IgE levels to Fel d 1 were significantly higher at each time point among children with symptoms to cat compared to asymptomatic children. Even though 81% of children sensitized to a single dog allergen molecule at 16 years had IgE reactivity to Can f 5, only about 10% of them reported symptoms. We calculated positive predictive values (PPV) for symptoms among subjects with IgE reactivity to cat or dog allergen extract or to the different pet allergen molecules. We observed that IgE to Fel d 1 and to cat extract had similar PPV for cat allergy. However, IgE to Can f 1 showed a higher PPV for dog allergy than IgE to dog extract. Thus, IgE testing with just one cat allergen molecule, Fel d 1, is as good as testing IgE to cat allergen extract and IgE to Can f 1 seems to have advantages over dog allergen extract. The results have implication for diagnosing cat and dog allergy.

Next we elucidated IgE reactivity to the pet allergens longitudinally in relation to symptoms. Only IgE to Fel d 1 at 4 and 8 years was significantly associated with increased risk of symptoms to cat at 16 yrs (OR=13.7, 95% CI 8.3-22.7 after adjustment for the other cat allergen molecules) (Fig. 5, lower part). With respect to dog allergen molecules, sensitization to Can f 1 at 4 and 8 years was the only dog allergen significantly associated with symptoms to dog at 16 years (OR=8.0, 95% CI 2.3-27.7 adjustment for other dog allergens)(Fig. 5, lower part). Furthermore, we noted that sensitization to more than one pet allergen at 4 or 8 years significantly increased the likelihood of having pet allergy symptoms at 16 years (Fig. 6). Thus, sensitization to Fel d 1 and Can f 1 at 4 and 8 years are risk markers for cat and dog allergy at 16 years.

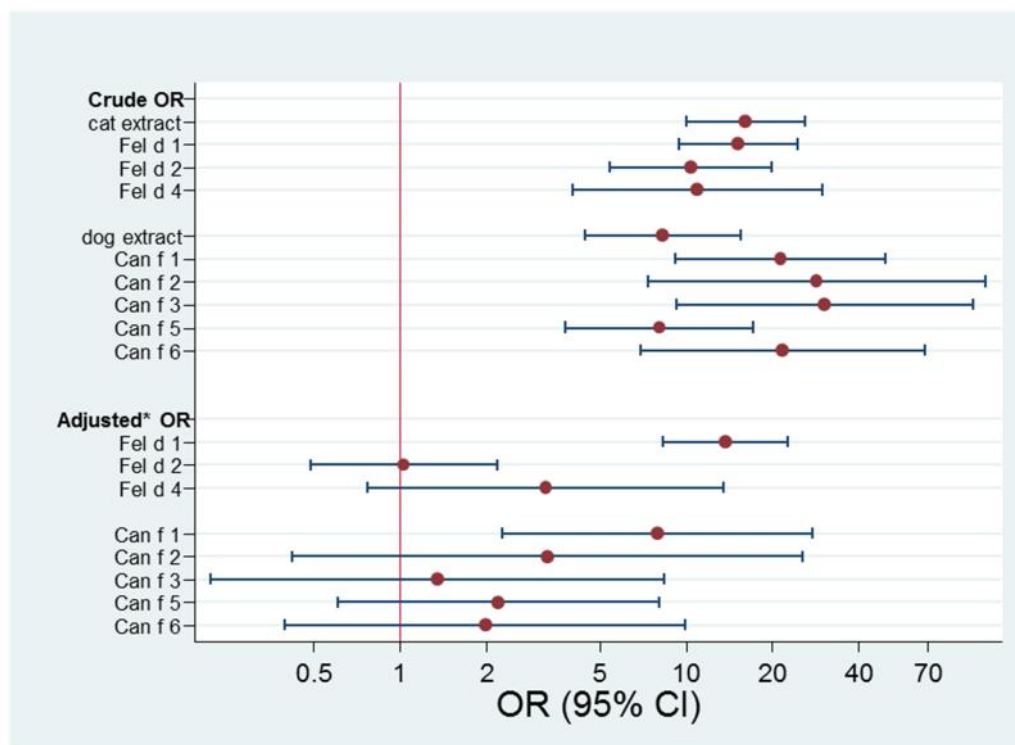


Figure 5 Longitudinal logistic regression. Crude and adjusted odds ratios (OR) for IgE sensitizations to each cat and dog allergen (ISU-E 0.3) at 4 and 8 years of age in relation to reported cat/dog allergy 16 years of age. N=779.

*Adjusted for concomitant sensitization to the other cat or dog components, respectively.

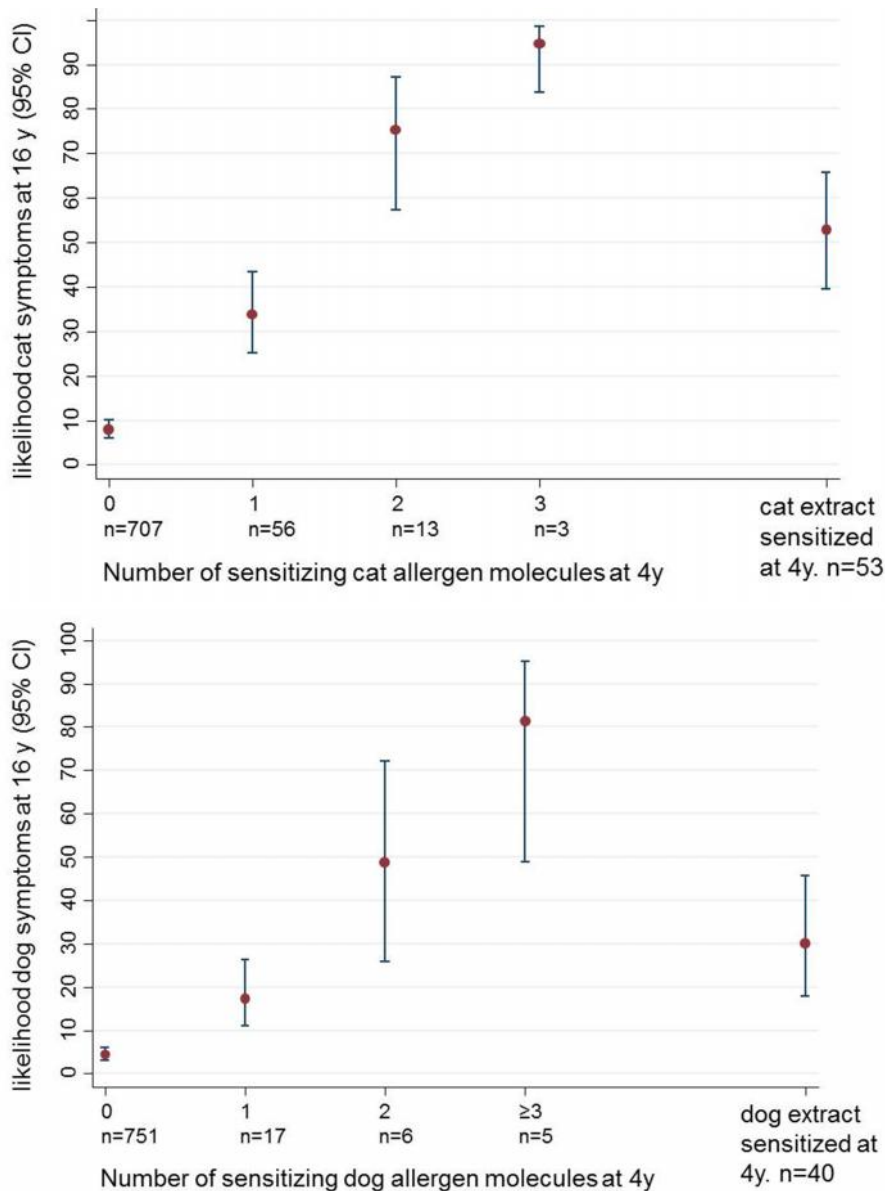


Figure 6 Likelihood (y-axis: percentage) of reporting symptoms to cat/dog at 16 years of age depending on the number of IgE-reactive cat (Fel d 1, Fel d 2, Fel d 4) or dog (Can f 1, Can f 2, Can f 3, Can f 5 and Can f 6) allergens and ImmunoCAP cat or dog extract sensitization (x-axes) at 4 years.

This is the first study to elucidate the usefulness of analyzing individual cat and dog allergen molecules as predictors of cat and dog allergy development from childhood to adolescence. For diagnosis of cat allergy, IgE to Fel d 1 is as good as IgE to cat extract and IgE to Can f 1 is superior to IgE to dog allergen extract. Thus, molecular-based allergy diagnostics may offer new opportunities for improving diagnosis of pet allergy and in particular allergy to dog.

4.4 DEVELOPMENT OF A MOUSE MODEL FOR CHRONIC ALLERGIC ASTHMA [IV]

In Paper IV, we aimed to develop a protocol for induction of chronic allergic disease in mice, in order to obtain a relevant model for studying novel treatment strategies for allergy to cat. Female BALB/c mice were presensitized with rFel d 1 adsorbed to Alum and subsequently challenged intranasally (i.n.) with cat dander extract (CDE) three consecutive days per week during five weeks in the main optimized protocol (Fig. 7). As a reference, one group of mice was subjected to the same protocol using chicken albumin (OVA) for sensitization and i.n. allergen challenge. rFel d 1 sensitized and PBS-challenged mice served as negative controls. Both the cat allergen and OVA models generated AHR in response to metacholine and inflammation as demonstrated by histological analyses of the lung tissue. The OVA model displayed typical signs of Th2 allergy with predominant influx of eosinophils and lymphocytes in BAL, and elevated levels of Th2 cytokines in lung tissue (IL-5 protein levels and IL-4 and IL-5 mRNA expression). In contrast, our cat allergen induced asthma model showed a mixed type of allergic response, with enhanced total numbers of lymphocytes, neutrophils and eosinophils in BAL, increased lung tissue IL-17a levels and elevated relative mRNA expression of IL-17a, IL-4 and IFN- γ in the lung tissue. In a separate protocol, AW inflammation and tissue remodeling was specifically studied by histological analyses of lung tissue. Both the cat allergen- and OVA sensitization and challenge protocols resulted in high inflammatory scores and both models exhibited signs of AW remodeling (Fig.8). Finally, the allergen-specific antibody response was of a mixed Th1/Th2 type in the cat allergy model, with elevated levels of IgE (not significant, $p=0.56$), IgG1 and IgG2a to Fel d 1 compared to PBS-challenged control mice.

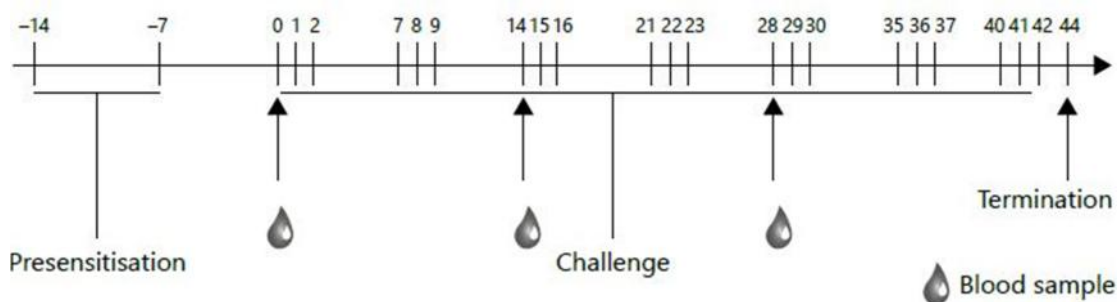


Figure 7 Presensitization (s.c. Alum-Fel d 1) and challenge (i.n. CDE) protocol.

We have previously developed an acute mouse model of allergy to cat.[118] The present model displays hallmarks of chronic allergic asthma, such as AHR, a mixed neutrophilic and eosinophilic infiltration in BAL similar to that in human allergic asthma, expression of the proinflammatory Th17 cytokine IL-17a and signs of remodeling in lung tissue. A chronic model for a chronic disease that is against a natural airway allergen, Fel d 1, and that more closely resembles natural i.n. exposure is a big advancement in allergy models. Earlier models were short acute models and/or with unnatural exposure by injections and/ allergens

like OVA. Mouse models with OVA are skewed towards producing Th1 cytokines that do not reflect human allergic, Th2, disease well [119]. This paper provides a relevant model for studying chronic allergic disease induced by a natural airway allergen. Thus, the model is suitable for testing novel strategies for allergy vaccination (i.e. ASIT) for evaluating and developing new treatments of human disease.

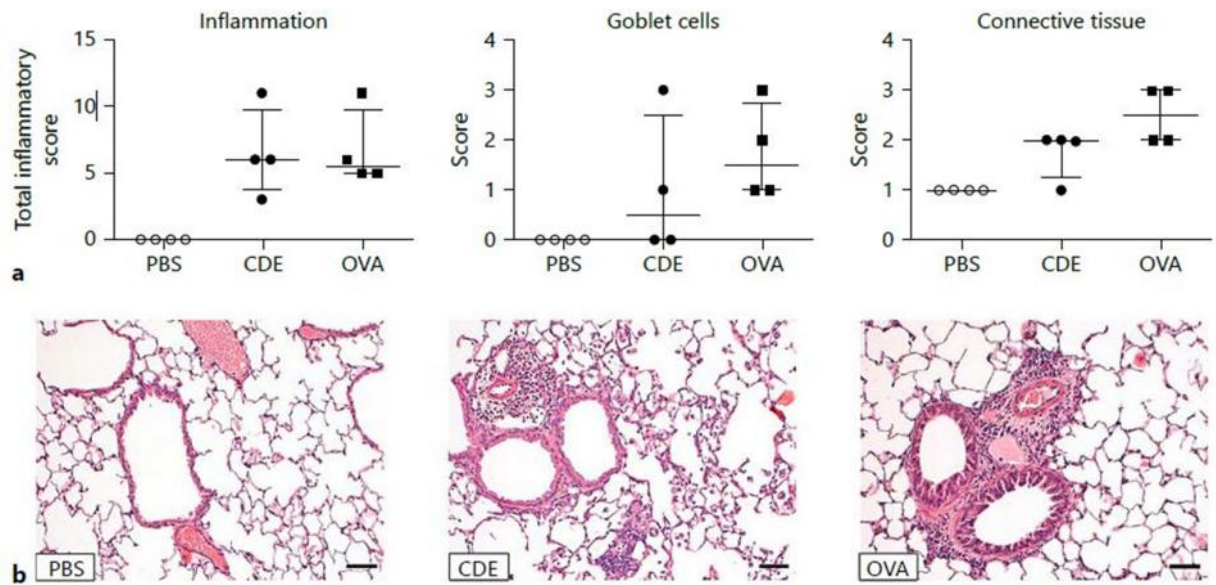


Figure 8 Inflammation and tissue remodelling in lungs from mice (n = 4). **a)** Total inflammation score based on the sum of scores (max score = 12) for the degree of inflammatory cell infiltration, amount of inflammatory infiltrate, and of macrophages and the area involved in lesions in HE-stained sections (left). Results from evaluation of PAS-stained sections measuring the number of goblet cells were estimated (middle) and Masson's trichrome-stained sections analyzing peribronchial and perivascular connective tissue was analyzed (right). **b)** Representative sections from PBS-, CDE- and OVA-challenged mice stained with HE.

5 CONCLUSIONS

Paper I: We evaluated dog saliva as a source of new allergen molecules for improved diagnosis of dog allergy. We found at least 12 protein bands in dog saliva that were recognized by IgE antibodies of dog-allergic patients, and several of those were not found in dog dander. This means that saliva has a greater diversity of IgE-binding proteins than dog dander, in which only a few IgE-binding proteins corresponding to already known dog allergens were detected. In dog saliva, four interesting new allergen candidates (BPIFA2, mucin-5B and angiopoietin-related protein 5-like (ANGPTL5) and IgA heavy chain constant region) were identified alongside known allergens. We also showed that current diagnostics for dog allergy, using dog dander extract, generate many false negative results. About one-fifth of patients with symptoms to dog, but lacking IgE antibodies to dog dander, were IgE positive to saliva.

Thus, dog saliva was shown to be a significant allergen source, and the shortcomings of dog dander extracts can be improved by adding dog saliva to the diagnostics of dog allergy.

Paper II: We investigated the novel allergen Fel d 7 and showed that it is a frequently recognized allergen in a Swedish cat sensitized population. IgE antibodies to Fel d 7 were found in 39% of cat sensitized patients and 89% of these were also sensitized to the major dog allergen Can f 1. Thus, cross-reactivity between these homologous lipocalin allergens was indicated. Fel d 7 was indeed shown to have a similar structure and to share B-cell epitopes with Can f 1. The biological activity of Fel d 7 was confirmed in basophil activation assays in cat allergic patients. We conclude that Fel d 7 may contribute to the co-sensitization noted in cat and dog allergic patients. Our data will be important for aiding diagnosis of allergy to dog and cat.

Paper III: We investigated the association between sensitization and symptoms to cat and dog allergens in children up to 16 years of age using the BAMSE birth cohort. The data showed that sensitization to Can f 1 and Fel d 1 during childhood was associated with significant increased risks of symptoms to dog and cat, respectively, at 16 years after adjusting for other dog/cat allergen molecules.

In addition, we found that IgE to Fel d 1 had similar positive predictive values (PPV) for cat allergy as cat extract did. However, IgE to Can f 1 showed a higher PPV for dog allergy than IgE to dog extract. Even though IgE to Can f 5 was more prevalent during childhood than Can f 1, it showed weaker associations to dog allergy. Thus, we showed how to interpret sensitization to individual cat and dog allergens and use thereof in pet allergy diagnostics.

Paper IV: We aimed to establish a mouse model for cat allergen-induced asthma that resembles human disease more closely than current cat allergy models. We could show that a protocol comprising pre-sensitization with Fel d 1/alum followed by repeated intranasal challenges with cat dander extract resulted in a model that displayed hallmarks of chronic allergic asthma. Furthermore, we demonstrated that the nature of the allergens used for sensitization and challenge in the model was critical for the outcome of the immune- and inflammatory response. While the commonly used model allergen OVA produced a typical Th2-type of response, we observed that i.n. administration of cat allergen extract generated a mixed response characterized by neutrophilic and eosinophilic inflammation and both Th2- and Th17-type cytokine production in the lung. The cat allergen induced asthma model will be a valuable tool for evaluating novel treatment strategies for cat allergy. In the longer run by improving ASIT regimes we hope that they will induce long lasting cure and diminish the need of symptomatic treatment.

6 FUTURE PERSPECTIVES

Future allergy diagnostics will be based on individual allergen molecules reflecting the natural allergen source. These allergen molecules will help to increase accuracy in allergy diagnosis and prognosis. In paper 1, we showed that dog saliva is a significant allergen source that should be added to dog dander for improved diagnostics of dog allergy. In the future, it would be of great interest to investigate the usefulness of dog saliva in other populations. Furthermore, whether children and adults with symptoms to dog have different IgE reactivity to dog saliva would be another valuable question to elucidate for increasing our knowledge regarding dog saliva in diagnosis. However, there are concerns with safety and protein stability of dog saliva due to degradation of proteins. Therefore, proceeding with the allergen candidates to identify novel proven allergens would be beneficial. Then, having had confirmed one or many new dog saliva allergens that could be cloned and produced recombinantly, would enable spiking dog dander extract with relevant recombinant dog allergens instead. The result of such an extract spiked with stable, characterized and well purified proteins would address the safety and stability issues and be able to be implemented in routine diagnostics, and in the future also for ASIT if we can improve the extract.

Cat allergy is a major problem worldwide. In paper II we show that Fel d 7 is an important cat allergen in a Swedish cat sensitized population. It would be of great interest to investigate its importance for asthma due to cat among children, which is an important pediatric problem. As Fel d 7 shares epitopes with the lipocalin Can f 1 it would be valuable to elucidate its role in cross-reactivity with lipocalins from other furry animals. Other lipocalins that are similar to Fel d 7 is one in Panda with 68% identity and horse and pig VEGP with 62% and 58% identity, respectively [76]. These have about as much sequence identity as Can f 1 and Fel d 7 do, and could be interesting in terms of cross-reactivity. The horse lipocalin is most relevant for further studies in pet allergies. This would increase our understanding of how cross-reactivity may translate into polysensitization.

Our 3D model reveals that the epitopes shared between Fel d 7 and Can f 1 are mainly exposed on the N and C terminus of the proteins. Since the antibodies to linear peptides only show linear surface antigens and cannot reach inside the structures or more complex conformational epitopes, where different non-linear sequences form a surface epitope, these would be interesting to explore. The 3D-model with the cross-reactive epitopes is a starting point for further studies into the allergens, and their exact structure should be determined by X-ray crystallography, where you also are able to visualize the interaction between conformational epitopes and antibodies.

Crystal structure and data on Fel d 7 will be important for further investigations in the structure and help in diagnostics, respectively. However, since Fel d 1 is so dominant the interest of Fel d 7 in diagnostics would mainly be in finding cat sensitization in the few Fel d 1 negative patients and in pet cross-reactivity patients. Further studies are needed to elucidate the clinical importance of Fel d 7.

Using recombinant and natural purified allergens in molecular-based allergy diagnostics, instead of extracts, offers not only new opportunities for improving diagnostics of allergy but also for determining prognosis. We show that IgE to Fel d 1 and Can f 1 in childhood are useful markers of cat and dog allergy in Swedish teenagers. Whether this holds true for birth cohorts from other countries needs to be elucidated. Such results are of major clinical importance with respect to treatment and avoidance. Our finding that some allergens, in particular Fel d 1 and Can f 1, can be used as strong prognostic risk markers for the development of allergy changes the future for doctors and ultimately patients to make wise decisions in pet allergen avoidance that could help protect people from developing allergies in the first place. One topic to follow up on is if information about early markers of future pet allergy, the allergen component sensitization in childhood, can improve the outcome in avoiding allergy development.

The presented mouse model for cat allergen-induced asthma exhibits hallmarks of chronic allergic asthma and will provide a relevant platform for development of new treatment strategies. As a next step, we would like to continue to test treatment regimes against cat allergy in this model. The effect of allergy vaccines may be enhanced by adding an adjuvant.

One candidate Fel d 1 vaccine that we would like to evaluate is Fel d 1 produced by virulence-attenuated *Listeria monocytogenes* (Lm). We have prepared a Fel d 1 vaccine candidate, in which Fel d 1 is carried by an Lm vaccine vector, Lm-Fel d 1, in this way. The gene for rFel d 1 has been cloned and transformed into Lm so that rFel d 1 is produced *in vivo* in the host receiving intraperitoneal immunizations. This novel vaccine candidate could be evaluated both in our previously established acute model for cat allergy [118] and more carefully in the new chronic mouse model of cat allergy. The stimulation from Lm could possibly be able to break the Th2 state of allergic disease since it is a strong immunomodulatory agent, which is complex but partly inhibits Th2 response and induces a Th1 response with IFN γ production.[120-123] Additionally, comparing the efficacy of Lm-Fel d 1 with the traditional ASIT adjuvant Alum, Alum-Fel d 1, could be performed side-by-side in our mouse models i.e. as in paper IV.

To conclude, this thesis is based on the application of pet allergen molecules, and spans from allergen characterization to prediction of symptoms and to the development of an allergy model for chronic airway disease. Paper I and II describe the usefulness of a forgotten allergen source from dog and the allergenic relationship between lipocalins from different allergen sources. Paper III shows how sensitization is linked to symptoms by analyzing multiple allergen components, implicating that diagnostics and prediction of future symptoms can be improved. Paper IV presents a new chronic mouse model of cat allergy for evaluation of new treatments. In the longer run, improving ASIT regimes will induce long lasting cure and diminish the need of symptomatic treatments.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Allergiska sjukdomar har beskrivits redan 900 e.Kr. men idag har allergiska sjukdomar blivit så vanliga att de har nått epidemiska proportioner. Allergen är vanligen ofarliga proteiner som vissa personer kan bli allergiska emot, dessa bildar antikroppar av IgE typ mot allergenet och sägs då vara sensibiliserade mot det allergenet. Runt 30 % av världens befolkning lider idag av minst någon allergisjukdom. Typiska allergiska symptom t. ex. rinnande näsa, röda ögon, hudreaktioner som klåda, eksem och nässelutslag, samt mer allvarliga symptom som astma och akuta anafylaktiska reaktioner, kan uppkomma när vårt immunförsvar försöker försvara oss mot ofarliga ämnen, allergener, i vår omgivning. Allergidiagnostiken baseras i stort på allergenextrakt, och för diagnostik av pälsdjursallergi på extrakt från pälsen. Extrakten är inte alltid så rena och kan vara svåra att standardisera, vilket får negativa effekter på tillförlitligheten. Därför finns det ett behov att vidareutveckla diagnostiken för att göra den mer tillförlitlig. Målet med denna avhandling är att förbättra diagnostiken för pälsdjursallergi och att utveckla en modell där nya behandlingsmetoder kan studeras.

I det första arbetet studerades hundsalliv som en källa till nya allergena molekyler för förbättrad diagnostik av allergi mot hund. Där visas att det finns minst 12 proteinband i hundsalliv som känns igen av allergiframkallande s.k. IgE-antikroppar från hundallergiska patienter. Hundpälsextrakt är inte tillräckligt bra på att diagnostisera hundsensibilisering i allergitester. Det är känt att tester för hundallergi kan ge falskt negativa resultat. I vårt arbete visade det sig att ungefär en femtedel av patienterna som får allergiska symptom av hund, men saknar IgE-antikroppar mot hundpäl, hade IgE-antikroppar mot hundsalliv. Hundsalliv visade sig vara en betydande allergenkälla som bör beaktas för att förbättra diagnostiken av hundallergi. Att kombinera hundmjäll och hundsalliv eller spetsa hundpälsextrakt med hundsalliv eller med allergen från alliv skulle vara fördelaktigt för att utveckla förbättrad hundallergidiagnostik.

I det andra arbetet i denna avhandling undersöks förekomsten av allergi orsakat av det nya kattallergenet "Fel d 7" i 94 katt-sensibiliserade patienter. Vi analyserade Fel d 7s allergiframkallande förmåga och dess förmåga att reagera på det liknade huvudallergenet i hund, "Can f 1". Mer än en tredjedel av de svenska kattsensibiliserade patienterna, 39 %, hade IgE antikroppar mot Fel d 7 och vi kunde visa att Fel d 7 är ett biologiskt aktivt allergen som utlöser allergi reaktioner i celler från kattallergiska patienter. Våra resultat visar att Fel d 7 är korsreaktivt med Can f 1. Det innebär att de två allergenerna har gemensamma ytstrukturer, vilket förmodligen är en bidragande orsak till att patienter ofta är sensibiliserade mot både katt och hund. Med hjälp av antikroppar riktade mot delar av Can f 1 kunde vi dessutom visa vilka ytstrukturer som Fel d 7 binder till i en 3D-modell som skapades baserat på en välstuderad mänsklig variant av proteinet.

I det tredje arbetet undersöks sambandet mellan sensibiliseringsmönster mot enskilda katt och hund allergenmolekyler under barndomen och allergisymptom mot dessa pälsdjur upp till 16

års ålder. Vi undersökte sensibilisering mot enskilda katt- och hund-allergenmolekyler i barndomen till puberteten med hjälp av BAMSE (Barn Allergi / Astma Mijö Stockholm Epidemiologi studien), en studie som har följt barn från de var 2-3 månader upp till 16 år. Blodprover och enkätdata från 779 slumpmässigt utvalda barn vid 4, 8 och 16 år användes. IgE antikropps-reaktivitet mot katt- och hund-allergenmolekyler analyserades med ett nytt diagnostiskt test, ett litet glas som innehåller allergenmolekyler från nästintill alla kända allergenkällor. Detta är den första studien som belyser nyttan av att analysera de individuella katt- och hund-allergen för att förutspå utveckling av allergi mot katt- och hund. För diagnos av kattallergi visade sig antikroppar mot huvud allergenet i katt ”Fel d 1” vara lika bra som kattextrakt. Huvud hund allergenet Can f 1 var överlägset hundallergenextrakt för att diagnosticera allergi mot hund. Således kan allergidiagnostik med de enskilda allergenen erbjuda nya möjligheter att förbättra diagnostiken för allergi mot sällskapsdjur, särskilt allergi mot hund. Att man kan använda diagnostiken för att förutspå risken för att utveckla katt- eller hundallergi i framtiden är av stor betydelse.

I det fjärde och sista arbetet presenterar en relevant sjukdomsmodell i möss för kattallergisk astma, som uppvisar drag av kronisk sjukdom i människa. Möss sensibiliserades med kattallergen och provocerades därefter med extrakt från kattpäls under fem veckor för att etablera allergi- och astmasymptom mot katt. Kattallergent är ett naturligt luftvägsallergen vilka inte har använts för lite i tidigare djurmodeller för allergiska sjukdomar. Den nya djurmodellen uppvisar inte bara akuta symptom som tidigare modeller utan även tecken på kronisk allergisk astma, som luftvägsöverreaktivitet, en inflammation i lungan som liknar den i mänsklig allergisk astma samt förändringar och förhårdningar i lungvävnad som karakteriserar kronisk astma. Det här arbetet har gett en relevant kronisk modell för att studera kronisk allergisk sjukdom. Således är modellen lämplig för att testa nya strategier för allergivaccination, för att utvärdera och utveckla nya behandlingar av allergisk sjukdom hos människor.

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quotation:

“If I have seen further than others, it is by standing upon the shoulders of giants.” –
Isaac Newton

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